Harris does make a blanket statement that there is little future for the use of rodent monoclonal antibodies for human therapy and he provides three reasons, 1) the short half-life, 2) poor recognition of rodent immunoglobulin constant region by human effector functions and 3) the human immune response against murine proteins. Harris et al fails to provide a context or basis for his statements. The statements are only general in nature.

Applicant submits that these general statements are not applicable to the claimed anti-selectin antibodies of the invention. Applicant has demonstrated that the El-246 antibody does not have a short half-life in mammals. Applicant has also disclosed that the antibody binds to human leukocytes, binds to human leukocyte lysates, binds to human E and L-selectin transfected cells, and binds to high endothelial venules from human tonsils. It is this binding, not the recognition of the immunoglobulin constant region, that is important for inhibiting the function of the cells. Therefore, the appropriate portion of the antibody molecule is interacting with the appropriate human cells.

In regard to possible formation of human anti-mouse antibodies, Applicant submits that this does not prevent the antibodies from acting as an effective anti-adhesion or anti-inflammatory agent. Applicants have demonstrated in vivo efficacy of the antibody in an animal model.

The facts do not support the Harris et al statement that there is little future for the use of rodent monoclonal antibodies in human therapy. Rodent monoclonal antibodies are currently being used on a routine basis in humans. For example, the murine monoclonal antibody OKT3, which reacts with human T cells, is being used to prevent acute renal-allograft rejection in

humans despite the fact that human anti-murine antibodies are produced in some patients. (Ortho Multicenter Transplant Study Group, The New England J. Med. Vol. 313, No. 6, pp 337-342, 1985 - Exhibit A).

The Examiner also quotes a statement from Harlan (1449, #4, Edgington, Biotechnology 1992, p 386, column 3, paragraph 4) to support the view for lack of utility of the claimed antibodies in humans. It is Applicant's view that Harlan's statement merely indicates that development of a commercial product is time consuming. Harlan's statement in no way suggests that humanized antibody, peptide, soluble receptor or saccharides lack utility under the meaning of §101. In fact, the overall paper has several positive statements for utility of anti-selectin therapeutics.

Paulson in referring to selectin-inhibiting SLe^x, states on page 385, 2nd column, paragraph 2 of the Biotechnology article,: "We are very excited... this means selectin-based therapeutics aren't just a pipe dream, but will come to fruition imminent..."

In the same article, Ahern states on page 388, third column, first paragraph: "... a treatment for acute adult respiratory distress syndrome would be of tremendous clinical benefit... Inhibiting lung E-selectin binding may prove useful in treating this disease that strikes thousands each year". Also in the same article on page 388, third column, second paragraph it states:

> Gage stalwartly defends the future of selectin-based therapeutics in chronic inflammatory disease. "Most of these diseases, such as asthma, are cyclical," he says. "If you can suppress the acute phase, you interrupt the cycle, and if you stop that, you curtail the whole process." A former drug-discovery executive for Hoffman La-Roche, he thinks the underlying rationale for selectin-inhibiting drugs is as sound as any he has seen.

Thus, the overall gist of the Edgington article in Biotechnology supports a utility of selectin inhibiting drugs in humans.

Applicant submits that in the art of anti-adhesion therapy, in vitro, ex vivo and in vivo animal models are accepted as predictive of efficacy in humans. The present invention discloses several "art-accepted" in vitro, ex vivo and in vivo assays. For example, page 43 of the specification discloses the use of the Stamper-Woodruff assay which is an ex vivo assay and is regarded by those in the art as replicative of adhesion interactions between lymphocytes and endothelium in vivo [see p. 45 Lasky et al 1992 Chapter 3, In: Adhesion, Its Role In Inflammatory Disease, J.M. Harlan and DY Liu (eds.) W.H. Freeman and Company, NY, pp. 43-63, Exhibit B]. Applicant has clearly shown in animal models the efficacy of the antibody of the present invention to prevent mortality in an ischemia/reperfusion model and the efficacy of the antibody to prevent lymphocyte homing.

The art recognizes that treatment using the in vitro, ex vivo and in vivo animal models as disclosed in the present specification are predictive of the outcome in humans.

An in vitro showing of pharmaceutical activity was held sufficient to meet the practical utility requirement of Brenner v. Manson, where structurally similar compounds had similar in vitro and in vivo activity. Cross v. Iizuka, 753 F.2d 1040, 224 USPQ 739 (Fed. Cir. 1985). Where the nature of the compound of composition is such that its properties are generally predictable from a knowledge of the compounds, the allegations of utility for such purposes need not be verified by clinical proof. (MPEP §608.01(p)).

Utility may be proved by in vivo or in vitro tests as would be convincing to those skilled in the art. In re Irons, 340 F.2d 974, 144 USPQ 351 (CCPA 1965). As stated in MPEP §608.01(p):

> [I]f the utility relied on its directed solely to the treatment of humans, evidence of utility, if required, must generally be clinical evidence, although animal tests may be adequate where the art would accept these as appropriately correlated with human utility. If there is no assertion of human utility, or if there is an assertion of animal utility, operativeness for use on standard test animals is adequate for patent purposes.

Thus, when an antibody has utility for both humans and other animals, the utility for such other animals is sufficient utility under 35 U.S.C. § 101. See In re Hartop, 311 F.2d 249, 135 U.S.P.Q. 419 (C.C.P.A. 1962).

The Examiner asserts that the specification fails to establish the utility of the claimed antibody for in vivo use in humans for four reasons.

These reasons are 1) that the antibody may be inactive before producing an effect; 2) the protein may not reach the target area; 3) other functional properties known or unknown may make the protein unsuitable for in vivo therapeutic use such as adverse side effects.

These reasons by the Examiner totally ignore the facts disclosed in the specification. Applicant has clearly and unambiguously shown using the lung ischemia/reperfusion animal model, that the antibody of the present invention produce a profound effect on treated animals. One hundred percent of the animals treated with the antibody of the present invention survived, whereas only 37.5% of the untreated animals and only 33.3% of the DREG 56 treated animals survived. It is obvious from these results that the antibody reached the target site, ie the lung

and treated it in an efficacious concentration. Moreover, the data shows that saturating levels of the antibody were maintained for 6 hours after a single bolus injection. Thus, the antibody of the present invention is not rapidly cleared from the circulation. Nor did the animals suffer from adverse side effects.

Proof of utility under §101 may be established by clinical or in vivo or in vitro data or combinations of these, which would be convincing to those skilled in the art. [In re Irons, 340 F.2d 924, 144 U.S.P.Q. 351 (CCPA 1965)].

Supplied herein is a declaration from John A. Steinberg, M.D. in support of utility of the claimed antibodies in methods for treating humans. As one skilled in the art, Dr. Steinberg declares that the results from the lung ischemia/reperfusion animal model using the EL-246 antibody are reasonably predictive of in vivo efficacy in humans. Dr. Steinberg declares that the inflammation-induced injury that occurs in the lung ischemia/reperfusion animal model corresponds with the inflammation-induced injury that occurs in lung transplantation and pulmonary injuries in humans. He further states that the EL-246 antibody was efficacious in treatment of the inflammation-induced lung ischemia/reperfusion injury in the animal model, as demonstrated by improved survival, inert gas shunt and respirator gas exchange. Most importantly, as one skilled in the art Dr. Steinberg declares that the efficacy of the El-246 antibody as demonstrated in the animal model is reasonably predictive of efficacy of the EL-246 antibody in humans. Applicant submits that if the asserted utility of the claimed subject matter is believable on its face to persons skilled in the art then the burden is upon the Examiner to give adequate support for rejections for lack of utility (MPEP 608.01(p)).

Claims 21-23 have been amended to recite additional specific characteristics or properties of the claimed antibodies. These claims are not pharmaceutical composition claims. Pharmaceutical compositions are covered by claim 8.

35 U.S.C. §112, First Paragraph Objection and Rejection

The specification was objected to, and Claims 1-33 were rejected for failing to provide an adequate written description of the invention, for failing to provide an enabling disclosure, and for failing to present a best mode for carrying out the invention.

The written description requirement is a separate requirement under § 112 and is distinct from the enablement requirement. However, the Examiner has not in Applicant's view, clearly distinguish his objections and rejections between these two requirements. Instead, the written description and enablement objection and rejections have been "lumped" together, which makes it difficult for Applicants to respond.

The description requirement requires that the applicant had in their possession, as of the filing date of the application, the specific subject matter claimed in the application. In order to establish a prima facie case of lack of written description, the Examiner must demonstrate that there is no express description corresponding to the invention as claimed, and that nothing is present in the specification or record to show how the description may be inferred by the person of ordinary skill in the art.

The specification fully complies with the written description requirement as prescribed under §112, first paragraph. Applicant would like to remind the Examiner that the claims form part of the specification. The claims are the original claims in the present application. Written

description, objections and rejections are appropriate under circumstances in which an Applicant has amended the original claims or added new claims to subject matter that is not described in the specification or in the original claims. Thus is not the case in the present situation. Applicants submit that the objection and rejection of claims are not properly based under the written description requirement section of §112, first paragraph, and should be rescinded.

In turning to the enablement issue under §112, first paragraph, the Examiner states that Applicant does not disclose how to use the antibodies of the present invention in humans, that those skilled in the art would not believe the operability of the claimed method and compositions for treating human inflammatory diseases, and that it would require undue experimentation to practice the invention. As stated in response under §101, those skilled in the art would accept the in vivo, ex vivo and animal models as predictive of efficacy in humans in the treatment of inflammatory diseases.

The Examiner states that it would require undue experimentation to practice the broadly claimed specificity of common antigenic determinants found on E-/L-selectins for therapeutic and diagnostic use following the teachings of the specification alone.

Applicant respectfully disagrees. The enablement provision of §112, first paragraph, ensures that one skilled in the pertinent art will be able to make and use the claimed invention without undue experimentation. Hybritech Inc. v. Monoclonal Antibodies Inc., 802 f.2d 1367, 231 U.S.P.Q. 81, 94 (Fed. Cir. 1986) cert. denied, 107 S.Ct. 1606 (1987). Enablement is not precluded by a necessity for some experimentation, such as routine screening, thus, the key word is "undue" not "experimentation". In re Wands, 858 F.2d 731, 8 U.S.P.Q. 2d 1400, 1404 (Fed.

Cir. 1988). Therefore, Applicants assert that given the present specification, those skilled in the art could easily, and as a matter of routine, screen for antibodies that react with a common antigenic determinant on both E-selectin and L-selectin and use the selected antibodies in the methods as claimed. The specification teaches one skilled in the art how to make and screen for the antibodies of the present invention. The specification discloses the desired characteristics of the antibodies of the present invention and the specification teaches the methods of using the antibodies in diagnosis and therapy.

The Examiner states that the EL-246 antibody is required to practice the claimed invention and that the specification does not provide a repeatable method for obtaining antibody EL-246. Applicant disagrees with the Examiner's view. Applicant would like to emphasize that he is the first to disclose that there are common antigenic determinants that are shared by different selectin molecules. One skilled in the art, advised of the teachings of the specification could make and screen for antibodies that bind a common antigenic determinant on E-selectin and L-selectin as a matter of routine. Applicants have disclosed the necessary immunogen and have disclosed the screening procedures for selecting the appropriate antibodies. Thus, Applicant should be entitled to the broad scope of the claimed subject matter.

Applicant submit that the present invention does not rely solely on EL-246 and as such deposit on EL-246 is not mandatory to meet the requirement under §112. Applicant must emphasize that antibodies that bind to a common determinant found on E- and L-selectin can be made and isolated without undue experimentation following the teachings of specification. Applicant has deposited the EL-246 hybridoma cell line that secretes the EL-246 antibody with

the ATCC as disclosed on page 15, lines 3-8 of the specification. Contained herein, is a Declaration by the inventor, Mark A. Jutila. Dr. Jutila declares that the hybridoma was deposited on May 22, 1992 with the ATCC under ATCC Accession No. HB11049 under the terms of the Budapest treaty. Applicant wishes to remind the Examiner that the reference to a biological material in a specification disclosure or the actual deposit of such material by the Applicant does not create any presumption that such material is necessary to satisfy 35 U.S.C. §112 or that deposit in accordance with these regulations is or was required [37 C.F.R. §1.82 (c)].

Applicant submits that all requirements under 35 U.S.C. §112 have been met. Reconsideration of this objection and rejection under 35 U.S.C. §112, first paragraph, is respectfully requested.

35 U.S.C. §112, First and
Second Paragraphs

Claims 10-17 and 26-29 were rejected under 35 U.S.C. §112, first and second paragraphs.

Claims 10-17 and 26-29 were rejected as indefinite for not defining the specificity of the antibody. Claim 10, 17 and 26 have been amended to recite the specificity of the antibody.

Applicant submits that claims 10-17 and 26-29 as amended clearly recite the specificity of the antibody for E-selectin and L-selectin. Applicant respectfully requests reconsideration and withdrawal of the rejection under 35 U.S.C. §112, first and second paragraphs in view of the amendments.

35 U.S.C. §112, Second Paragraph Rejection

Claims 1-33 were rejected under 35 U.S.C. §112, second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claims 1, 3, 4, 6, 8, 18-25 and 30-33 were rejected as indefinite for the recitation of "common antigenic determinant." Applicant respectfully submits that this recitation particularly points out and distinctly claims the specificity of the claimed antibody. It is clear from the specification that the phase refers to an epitope which is found on both E-selectin and L-selectin.

Claims 1, 3, 4, 6, 8 and 18-33 were rejected for the recitation of "capable of recognizing" and "capable of inhibiting". Claims 1, 24, 25 and 26 have been amended to recite "which specifically binds" and claims 21 and 23 have been amended to recite "specifically inhibits" as suggested by the Examiner.

Claim 2 was rejected as indefinite or the recitation of "EL 246". Claim 2 has been amended to recite "EL-246".

Claims 8 and 9 were rejected as indefinite in the recitation of "suspecting". Claim 8 has been amended to recite "suspected".

Claims 10-17 were rejected as indefinite in the recitation of "with the intent of reducing tissue damage". Claims 10 and 17 have been amended to replace "with the intent of reducing" with "to prevent or inhibit".

Claim 29 was rejected as indefinite in the recitation of "similar". Since claim 26, from which claim 29 depends, has been amended herein to recite the specificity of the monoclonal antibody. Claim 29 has been amended to recite the EL-246 antibody.

Claims 30-31 were rejected as indefinite in the recitation of "endothelial cell layer". The Examiner states that "endothelial cells" is more appropriate. Applicant respectfully disagrees. "Endothelial cell layer", as clearly defined by the specification, is a layer of endothelial cells that line arteries, veins, capillaries, lymphoid vessels and the like (page 12, lines 5-6; page 28, lines 27-31), i.e. the endothelium (page 2, lines 17-22; page 43, lines 27-34; page 44, lines 1-3; page 62, lines 1-10 and lines 23-27; page 64, lines 30-34). Thus, the recitation, read in light of the specification, clearly and unambiguously defines the subject matter of the claimed invention.

§102(f) Rejection

Claims 1-8 and 19-25 were rejected under 35 U.S.C. §102(f) for the Applicant allegedly not inventing the claimed subject matter. Applicant, Mark A. Jutila, has filed herein a Declaration under §1.131 which states that he is the sole inventor of the subject matter disclosed in the present patent application, as well as the sole inventor of the subject matter disclosed in the parent application, U.S. Serial No. 07/887,695 filed May 22, 1992.

The declaration clearly states that the coauthors of the publication in J. Exp. Med. Vol 175:1565-1573 (June 1992), i.e. Watts, Walcheck and Kansas are not coinventors of the subject matter disclosed and claimed in the present application. The declaration states the contribution to the publication of each of the coauthors. The declaration points out that the contribution of Watts, Walcheck and Kansas to the publication was technical and not inventive in nature. The

declaration also points out that the parent application which was filed May 22, 1992 disclosed the subject matter reported in the J. Exp. Med. article in June of 1992. Mark A. Jutila is the sole inventor of the parent application.

Thus, the facts clearly show that Mark A. Jutila is the sole inventor of the subject matter disclosed and claimed in the present application. Applicant respectfully requests withdrawal of the rejection and §102(f).

35 U.S.C. §102(b) Rejection

Claims 1, 6, 18, 19 and 21-25 were rejected under 35 U.S.C. §102(b) as anticipated by Kishimoto et al (PNAS, 1990) as evidenced by Jutila et al (J.Exp. Med., 1992). The action states that Kishimoto et al teach the DREG-56 antibody which is described as a L-selectin specific antibody. The office action goes on to state that "Kishimoto et al did not recognize its E-selectin specificity". To justify this statement, the action points to Jutila et al as allegedly showing evidence that the DREG-56 antibody has the same or nearly the same binding specificities as the exemplified EL-246 monoclonal antibody. Moreover, the action states that the claims do not recite the particular limitations that distinguish between DREG-56 and EL-246.

Applicant respectfully traverses this rejection. The claimed subject matter is a monoclonal antibody that specifically binds a antigenic determinant common to both E-selectin and L-selectin. Kishimoto et al fail to anticipate an antibody that binds to both E-selectin and L-selectin.

The Examiner's interpretation that the DREG-56 has specificity for E-selectin is mere opinion on the part of the Examiner and is not supported by the facts contained in both Kishimoto et al and in Jutila et al.

As one skilled in the art, Kishimoto et al clearly and unambiguously state that the DREG-56 antibody has specificity for L-selectin and that it does not have specificity for E-selectin. Kishimoto et al tested their antibodies for reactivity to E-selectin. None of the five DREG monoclonal antibodies, including DREG-56 bind to E-selectin.

Page 2247-2nd column, first paragraph Kishimoto et al stated:

> COS cells transfected with the LAM-1 cDNA, but not with the structurally related ELAM-1 cDNA, react with LAM-1, Leu-8, TQ1, and DREG-55, -56, -110, -152, and -200 mAbs.

LAM-1 cDNA is L-selectin cDNA and ELAM-1 cDNA is E-selectin cDNA. Thus, Kishimoto et al clearly presents facts that prove that the DREG-56 antibody is specific for and binds to L-selectin and not E-selectin. The DREG-56 antibody does not recognize a common antigenic determinant present on both E-selectin and L-selectin a required by the claims. In contrast to DREG-56, the antibodies of the present invention do react with cells transfected with E-selectin cDNA (page 39, Example 2).

Jutila et al cannot be used as support for the Examiner's opinion that DREG-56 has the same or nearly the same binding specifics as EL-246. Jutila et al clearly teaches that the DREG-56 antibody does not have the same specificity of the antibody disclosed and claimed in the present invention.

The Examiner points to Figure 7 of Jutila et al to support his view. Figure 7 demonstrates that the antibody EL-246 of the present invention, ie inhibits human lymphocytes from binding peripheral lymph node HEV (L-selectin mediated binding) (Figure 7A). The antibody of the present invention, ie, El-246 also inhibits neutrophil adhesion to E selectin cDNA transfected L cells (E-selectin mediated binding) (Figure 7B). DREG 56 antibody was shown to inhibit human lymphocytes from binding HEV (L-selectin mediated binding) (Figure 7A). However, DREG 56 was not used in the E-selectin assay in Figure 7B. Did the Examiner ever consider why this was the case? Jutila et al state on page 1566, 2nd column, line 8 that the DREG 56 antibody recognizes human L-selectin, citing to the Kishimoto PNAS article. As stated above, Kishimoto provides facts that all five of the DREG antibodies including DREG 56 do not bind to E-selectin.

The Examiner has also pointed to page 1571, column 1, lines 306 to argue that the DREG 56 antibody is the same as the present invention. This statement merely indicates that EL-246 and DREG 56 stain L-selectin expressing cells as detected by two-color flow cytometric staining (see page 1568, 2nd column, lines 3-6).

Jutila et al teach that the EL-246 antibody is unlike any other previously reported antiselectin antibody. On page 1571, column 1, it states: "Even though many antiselectin mAbs have been developed, none have been shown to have the staining characteristics of EL-246".

For a proper rejection under 102(b) the cited art must disclosed the invention exactly as claimed in the present application. Kishimoto et al does not disclose an antibody that binds to a common antigen determinant on E-selectin and L-selectin. Contrary to the Examiner's view,

Jutila et al support the Applicant's position showing that the El-246 antibody is distinct from the DREG 56 antibody. Moreover, the Examiner has admitted that Table 1 in Jutila et al teaches "some distinctions" between the DREG 56 and EL-246 antibodies.

Thus, the facts contains in both Kishimoto et al and in Jutila do not provide a proper basis for the rejection under §102(b) and should be withdrawn.

35 U.S.C. §103 Rejection

Claims 1-33 were rejected under 35 U.S.C. §103 as being unpatentable over Kishimoto et al in view of Lasky et al U.S. Patent No. 5,098,833, Bevilacqua et al U.S. Patent No. 5,081,034, and Watson et al Nature, 1991. In addition, Jutila et al J. Exp. Med. was provided as pertinent art of record.

In terms of Jutila et al, Applicant has filed a §1.131 declaration stating that Mark A. Jutila, a coauthor of the publication, is the sole inventor of the subject matter disclosed and claimed in the present application that was filed less than one year after the publication. Therefore, Jutila et al may not be used as a basis for rejection under §103.

As stated above under §102, Kishimoto et al disclose that the DREG-56 antibody does not bind to E-selectin. This is a fact clearly presented by data in Kishimoto et al. All 5 DREG antibodies produced by Kishimoto et al fail to bind with E-selectin. Kishimoto et al only teach or suggest antibodies specific for L-selectin. Further, Kishimoto et al does not teach or suggest that E-selectin and L-selectin may share a common antigenic determinant. Thus, one skilled in the art would not be motivated to attempt to raise antibodies that bind to a common antigenic determinant on E-selectin and L-selectin.

Lasky et al '833, Bevilacqua et al '034 and Watson et al 1991 alone, or in combination, fail to correct the deficiencies of the primary reference.

Lasky et al '833 was cited as teaching the cloning of L-selectin and its use in the diagnosis and treatment of inflammatory diseases. Lasky et al '833 fail to teach or suggest antibodies that bind to a common antigenic determinant on both E-selectin and L-selectin. In fact, Lasky et al '833 does not even mention E-selectin.

Bevilacqua et al was cited as teaching the cloning of E-selectin and its use in the diagnosis and treatment of inflammatory diseases. Bevilacqua et al fail to teach or suggest antibodies that bind to a common antigenic determinant on E-selectin and L-selectin. Bevilacqua et al fail to make any mention of L-selectin.

Watson et al was cited as teaching the use of L-selectin specific molecules as therapeutic agents to inhibit viral infection or immune function. Applicants disclose and claim antibody having dual specificity for two different cell types, L-selectin and E-selectin expressing cells and methods of inhibiting the function of these two different cells using the antibody.

In contrast, Watson discloses soluble L-selectin for inhibiting the function of L-selectin expressing cells. Watson does not propose that the soluble L-selectin molecule is effective in inhibiting any selectin-expressing cells other than L-selectin expressing cells. In fact, Watson's suggestion that combinations of soluble adhesion molecules may be required for therapy implies that each soluble L-selectin is specific against L-selectin expressing cells, soluble E-selectin is specific against E-selectin expressing cells, etc.

Therefore, Watson et al in no way teaches or suggests that L-selectin and E-selectin share a common epitope. There is no analogy that can be drawn from Watson's suggestion of combinations of soluble selectins for treatment of inflammatory responses and the present invention of one antibody that inhibits the function of two different selectin bearing cells.

The Examiner has invited the Applicant to make a factual showing that the antibody of the present invention is different from DREG-56. Applicant submits that the Examiner has failed to provide a prima facie case of obviousness and as such, Applicant is not required to make such a factual showing. Moreover, the facts that distinguish the antibody of the present invention from DREG-56 are very clear from the disclosure in Kishimoto et al. Kishimoto et al provides data that proves that DREG 56 does not bind to E-selectin. Thus, the antibody of the present invention is novel and nonobvious in view of Kishimoto et al as the antibody of the present invention binds to E-selectin and L-selectin.

Further, the information provided in Table 1 of Jutila et al, which corresponds to Table 1 of the present application demonstrates the differences in specificity of the antibody of the present invention and not to similarities with DREG 56. The table shows that the epitope recognized by EL-246 is different from the epitope recognized by DREG 56. Further, the data shows that the epitope recognized by EL-246 is found on L-selectin from five different species of animals, whereas the epitope recognized by DREG 56 is only found on L-selectin from two species of animals.

Applicant respectfully requests reconsideration and withdrawal of the rejection under §103.

Favorable action by the Examiner is earnestly solicited.

Respectfully submitted,

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CHAPTER 3

The Homing Receptor (LECAM 1/L Selectin): A Carbohydrate-Binding Mediator of Adhesion in the Immune System

Laurence A. Lasky

Leukocytic or white blood cells, given their role as protectors of the organism, must be highly mobile so that they are able to migrate quickly to regions of insult in order to deal with invading pathogens. These cells must then undergo a hasty metamorphosis that results in a change from a mobile state to a highly adhesive one. These adhesive cells then adhere to the endothelium adjacent to regions under attack, after which they become activated advocates of the immune system, resulting in migration toward and ultimate destruction of the pathogen. Thus, these cells must maintain, through a complex combination of gene expression and protein regulation, either nonadhesive or adhesive states at appropriate times in order to insure the highest degree of immunologic surveillance to the organism.

The importance of this so-called inflammatory response is underlined by the very limited life expectancy of individuals that have one or more defects in this protective pathway. For example, individuals with the leukocyte adhesion deficiency (LAD) syndrome have a simple defect in the synthesis of one of the molecules involved in the adhesive interaction between leukocytes and the endothelium, the beta 2 integrin chain.[1–4] The absence of this single

protein component causes a lack of cell surface heterodimeric integrin expression and results in the inability of neutrophils to properly adhere to the vessel wall and migrate to sites of, for example, bacterial infection. This often leads to a series of virulent bacterial infections that can cause the early death of the individual. These findings underline the importance of the inflammatory response to protection of the organism and are consistent with the critical role of leukocyte adhesion in this protective response. In addition, they have stimulated a number of animal studies[4a] that indicate a clinically relevant role for antibodies directed against leukocyte integrin subunits.

In the 1980s, an explosion in knowledge regarding the cell and molecular biology of leukocyte adhesion occurred. Perhaps the most interesting aspect of these discoveries was a description of three of the families of molecules involved with leukocyte adhesion during the inflammatory response.[4] The first of these families, mentioned above, is encompassed by the heterodimeric alpha and beta integrin chains. The second of these families corresponds to some of the glycoprotein ligands for the integrins and has been referred to as the *immunoglobulin superfamily of adhesion molecules* (see Chapter 1 for a review of these two families). The final, and most recently discovered, family is unique among cell adhesion molecules because it accomplishes adhesion by means of protein–carbohydrate, rather than protein–protein, interactions. This family, the LECCAM (lectin cell adhesion molecule) or selectin family, contains three members: the homing receptor (LECAM 1, L selectin), ELAM-1 (LECAM 2, E selectin) and GMP-140/PADGEM (LECAM 3, P selectin).[5,6] While molecular descriptions for all three of these glycoproteins were communicated virtually simultaneously, the paradigm for this unique type of adhesion molecule, and the subject of this chapter, is the homing receptor.

HISTORICAL PERSPECTIVE

One of the most important trafficking pathways of the immune system is that traversed by B and T lymphocytes as they migrate from the bloodstream to the peripheral lymphoid tissue.[7–13] This is a critical migratory conduit, as it allows for cells with a diverse spectrum of antigenic specificities to encounter, with the highest probability, external, and often harmful, antigenic stimuli. Early *in vivo* experiments by Gowans, Ford and others suggested that lymphocytes isolated from peripheral lymphoid tissues tended to migrate back to this type of lymphoid organ, while those cells taken from other lymphoid regions tended to migrate back to these realms, implying some type of organ-specific migration or "homing" capacity.[4,9] It was theorized early on that the induction of this organ- or region-specific homing might have been due to adhesion molecules on the lymphocyte cell surface that were specialized

to adhere to ligands expressed only in one type of lymphoid organ or another.[13] However, the relatively primitive state of cell and molecular biology and immunology at that time prevented an accurate molecular description of such adhesion molecules, and the observation of organ-specific homing remained an interesting, although poorly explained, phenomenon.

Two major breakthroughs allowed for the description of at least one of the molecules involved in organ-specific lymphocyte homing. The first consisted of an *in vitro* assay that replicated the adhesive interactions between lymphocytes and the endothelium of lymphoid organs. In this assay, named the Stamper-Woodruff assay after its developers, frozen sections of various lymphoid organs were incubated with lymphocytes, the sections were washed, and the degree of specific binding between the added lymphocytes and the specialized high walled endothelium of the postcapillary venules of these organs was determined.[14,15] An interesting initial finding with this assay was that the adherence of lymphocytes to peripheral lymph node endothelial venules was entirely calcium-dependent (see below). In addition, the resultant adhesive interactions seemed to specifically represent those found *in vivo,* and, therefore, allowed for the development of immunologic reagents that could detect the adhesive molecules involved with these specific interactions. This led to the second breakthrough, where a rat monoclonal antibody, termed the *MEL-14 antibody,* directed against a 90-kilodalton (kD) glycoprotein on the murine lymphocyte cell surface, was found to specifically block the adherence of lymphocytes to the postcapillary venule endothelium of peripheral, but not Peyer's patch (gut), lymphoid organs.[16,17] This result demonstrated two important concepts. The first was that a specific glycoprotein on the lymphocyte cell surface was, at least in part, responsible for the adhesion of these cells to the lymphoid endothelium. The second was that this adhesion appeared to be specific for one type of lymphoid organ, the peripheral lymph node, suggesting that the ligand for this adhesion molecule may have been expressed in a lymphoid tissue-specific manner. The latter result was consistent with previous theories of potential mechanisms for organ-specific homing and led to the name "homing receptor" for the 90-kD adhesive glycoprotein recognized by the MEL-14 monoclonal antibody.

At this time, Rosen, Stoolman and colleagues were utilizing the same *in vitro* cell-binding assays to investigate the possibility that the adhesion between lymphocytes and peripheral lymphoid endothelium was due to protein–carbohydrate interactions. While considered somewhat heretical at the time, these investigators provided clear evidence in the early 1980s that some charged monomeric carbohydrates, such as mannose-6-phosphate, were able to specifically inhibit the adhesive interactions between lymphocytes and peripheral lymph node endothelium, although these carbohydrates were effective only at millimolar concentrations.[18] Such inhibition was not found with Peyer's patch endothelium, consistent with the possibility that a different

adhesion system was utilized in this lymphoid organ. These investigators went on to show that polymers of mannose-6-phosphate (polyphosphomannan ester or PPME) or fucose-4-sulfate (fucoidin) from natural sources (yeast and algae, respectively) were effective inhibitors of lymphocyte-peripheral lymph node (lymphocyte-pln) endothelium binding at much lower concentrations (micromolar), further strengthening the concept that carbohydrates may have been involved in this adhesive interaction.[19,20] It was also shown that treatment of the lymphocyte, but not the endothelium, with such blocking carbohydrates inhibited adhesion, consistent with the expression of a carbohydrate-binding molecule on the lymphocyte, but not endothelial, cell surface. In addition, fluorescent beads coated with PPME were found to bind to the lymphocyte, further strengthening the possibility that the carbohydrate-binding receptor was on the lymphocyte surface. The calcium dependence of this PPME bead binding was noted as an interesting correlation with the calcium dependence of lymphocyte–endothelial binding in the Stamper-Woodruff assay.[21] Finally, treatment of peripheral lymph node, but not Peyer's patch, sections with the sialic acid-removing enzyme sialidase (neuraminidase) inhibited the endothelial binding of lymphocytes *in vitro* and *in vivo*,[21a] consistent with the possibility that this interaction was, at least in part, mediated by the carbohydrate sialic acid.

The results of the blocking of peripheral lymph node endothelium binding by lymphocytes with both the MEL-14 monoclonal antibody and carbohydrates suggested a possible relationship between the 90-kD homing receptor recognized by MEL-14 and a potential carbohydrate-binding adhesion molecule on the lymphocyte cell surface. The MEL-14 antibody and carbohydrate-blocking studies were united when it was shown that this antibody could block the binding of PPME-coated beads to the lymphocyte cell surface. It was also shown that cells selected for high levels of MEL-14 antigen expression also bound higher levels of PPME and adhered to pln endothelium more dramatically.[22] These results were consistent with the possibility that the 90-kD antigen recognized by the MEL-14 antibody bound to carbohydrates such as PPME and, because both the antibody and carbohydrates blocked lymphocyte–endothelial binding, that this glycoprotein adhered to the endothelial surface by means of protein–carbohydrate interactions. While this idea was extremely provocative, the field awaited the next major step toward demonstrating the role of carbohydrate recognition in lymphocyte–endothelium adhesion: the cDNA cloning of the homing receptor.

cDNA CLONING OF THE MURINE AND HUMAN HOMING RECEPTOR

N-terminal micro-sequencing of homing receptor antigen isolated by MEL-14 antibody affinity chromatography allowed two groups to simultaneously

clone the cDNAs encoding the murine homing receptor.[23,24] Comparison of the encoded amino acid sequence with previously reported protein sequences revealed that the entire extracellular domain of the homing receptor was constructed from a number of motifs that were derived from other proteins. The N-terminus of the homing receptor encoded a signal sequence utilized for protein secretion into the endoplasmic reticulum. Immediately following this domain was a 116-amino acid motif that was homologous to the type C or calcium dependent carbohydrate binding proteins or lectins. While the overall homology in this region with these other lectins was relatively low (~25%–30%), there was a high degree of conservation of a subset of amino acids (the so-called "Drickamer motif" residues[25]) that are predominant in a diversity of calcium-dependent lectins. In addition, the disulfide bridges were also conserved with other lectins in this family. These homology comparisons, in addition to the previous hypotheses concerning the possible relationships between the homing receptor and a carbohydrate-binding receptor, were thus consistent with a physiologically important, carbohydrate-binding role for this domain. Following this motif was a 33-amino acid region that was homologous to the epidermal growth factor motif found in a wide variety of proteins. The degree of conservation here was also quite low, but potentially structural amino acids such as cysteines and glycines were highly conserved. Following this motif were two identical copies, at both the amino acid and nucleic acid level, of a 62-amino acid motif homologous to the short consensus repeat found in a wide array of complement-binding proteins.[26] This region was followed by a short region that led to a highly hydrophobic transmembrane anchor domain. Finally, a short, charged cytoplasmic domain was seen. In summary, the homing receptor was found to contain a number of protein motifs; potentially the most interesting and relevant is an N-terminal domain highly related to carbohydrate-binding (lectin) proteins.

The discovery of a C-type (calcium-dependent) lectin at the N-terminus of the homing receptor vindicated previous carbohydrate blocking data and, together with this data, provided the first clear evidence that cell adhesion in the immune system may be accomplished by protein–carbohydrate interactions. In addition to the carbohydrate blocking studies, these results were entirely consistent with the previously-noted calcium dependence of lymphocyte–lymph node endothelium and lymphocyte–PPME binding, as the carbohydrate binding by this type of lectin is entirely calcium-dependent. Indeed, at this time, the data were entirely consistent with this adhesion being solely due to the interaction of the homing receptor lectin domain with an endothelial-specific carbohydrate(s). As discussed below, however, the problem is somewhat more complicated than this. Suffice it to say, however, that what began as simple experiments demonstrating blocking of cell adhesion with monomeric sugars led to the discovery of a new type of adhesion molecule that appeared to regulate lymphocyte adherence through protein–carbohydrate interactions.

In contrast to the clear-cut results that were obtained with the MEL-14 antibody in the murine system, the identity of the human homing receptor was quite confusing. Butcher and colleagues reported in a series of papers that an antigen that they identified as the Hermes glycoprotein by antibody studies was the human equivalent of the murine homing receptor.[7] This identity was based on similar molecular weights (~90 kD), the ability to pre-clear murine homing receptor with a polyclonal antibody against the Hermes glycoprotein, and adhesion blocking studies using the previously described frozen section assay. This latter assay gave somewhat conflicting results depending upon the type of lymphoid tissue examined and the nature of the anti-Hermes antibody (monoclonal or polyclonal) used for the blocking studies. In addition, the tissue distribution of the Hermes glycoprotein was completely different from that found previously for the murine homing receptor, raising further doubts about its homology with the murine homing receptor. As with so many confusing issues in cell biology, the issue was clarified by the molecular cloning of the cDNAs encoding the human Hermes antigen and the human equivalent of the murine homing receptor.[27–30] These data indicated that the preliminary data concerning the homology of the Hermes glycoprotein and the murine homing receptor were incorrect. Thus, the Hermes antigen was found to encode a completely different glycoprotein that was homologous to cartilage link proteins and that was subsequently shown to be a hyaluronic acid receptor. This conclusion did not support the hypothesis that the Hermes antigen was a specific homing receptor, but instead was consistent with the possibility that this protein may function in a more nonspecific manner in cell adhesion. In contrast, the human homologue of the murine homing receptor was found to bear a high degree of sequence homology with its murine counterpart. The human receptor contained a lectin domain, an epidermal growth factor (egf) domain, two nonidentical copies of a complement binding-like motif, a transmembrane anchor, and a short cytoplasmic tail. The relative homologies were quite high in the lectin and egf motifs (~85%) and somewhat lower in the complement binding-like motifs (~70%–80%). Interestingly, the transmembrane domain showed almost complete homology between the human and mouse sequences (1 amino acid change in 39 residues [~97%] include regions before and after the ~20 residue transmembrane motif), suggesting that this region may fulfill more complex functions than mere plasma membrane anchoring. In addition, the cytoplasmic tail of both the human and murine homologues both appeared to contain at least one potential protein kinase C-dependent phosphorylation site, consistent with the possibility that this high degree of conservation in the transmembrane and surrounding regions may have been involved with interactions with kinases. The high degree of overall sequence homology between the human and murine homing receptors was consistent with previous trafficking and cell-binding assays that demonstrated a prominent level of conservation

between the lymphocyte adhesive and migratory mechanisms of human and mouse. In conclusion, it appeared that the adhesive pathway mediated by the homing receptor was a relatively early physiologic solution that remained little changed during the evolution from mouse to man.

An additional important finding derived from the cloning of the human homing receptor was the demonstration that it was homologous to a previously described lymphocyte surface antigen of unknown identity that was recognized by the Leu 8 and TQ 1 monoclonal antibodies.[28,31] These results were interesting because they allowed for a reappraisal of the previous work done with these antibodies. This work suggested that different T-cell subsets appeared to express different levels of the homing receptor.[32,33] For example, the naive subset appeared to show uniformly high expression of the homing receptor while the memory subset appeared to show a bi-modal distribution of expression.[31,33] These results were consistent with the possibility that the homing receptor mediated trafficking of different T-cell subsets, depending upon their phenotype. More recent work with the Leu 8 antibody has shown that skin lymphocytes appeared to also express high levels of this adhesion molecule, suggesting a possible role for this glycoprotein in skin trafficking. Additional antibodies against the human homing receptor have been produced (i.e., the LAM and DREG series), and results with these monoclonal antibodies have been generally consistent with those found using the previously identified monoclonals.[34–37] One notable difference, however, appears to be the expression of the homing receptor on thymocyte populations of mouse and humans. In the case of murine thymocytes, the mature thymocyte population appears to specifically express the homing receptor (i.e., are MEL-14 positive),[16] while in humans, both mature and immature populations express the antigen.[31] While much of this work is phenomenologic, it is consistent with a potential role for this receptor in directing different cell types to different lymphoid and inflammatory tissue compartments (see below).

As has been reviewed elsewhere,[5,6,38] the homing receptor was found to be a member of a family of adhesion molecules that all seemed to utilize protein–carbohydrate interactions to mediate adhesion, and has been variously termed the *LECCAM* or *selectin* family. The second member of the family, the endothelial leukocyte adhesion molecule (ELAM), was found to have a very similar overall mosaic construction as the homing receptor, with the exception that this glycoprotein had six complement binding-like repeats. This molecule was found on the endothelium, bound all leukocytes to varying degrees, and was inducible by inflammatory mediators such as interleukin-1 or tumor necrosis factor. The third family member, GMP-140 or platelet activation-dependent granule-external membrane protein (PADGEM), also had a similar structure with the exception that it had eight to nine complement binding-like repeats. In addition, there appeared to be a soluble form

of this glycoprotein that was missing the transmembrane domain, probably removed by mRNA splicing. This glycoprotein was found in platelet alpha granules and the Weibel-Palade bodies of endothelial cells. In both cell types, the protein could be rapidly expressed on the cell surface by thrombin activation of platelets or the endothelium, where the adhesive character of the glycoprotein endowed cells expressing it with the ability to bind neutrophils and monocytes. The overall degrees of sequence homology between these glycoproteins was high in the lectin and egf domains (~65%–70%) and somewhat lower in the rest of the molecules (~40%). A ligand for both ELAM and PADGEM/GMP-140 appears to be the tetrasaccharide sialyl Lewis-X[39,40] (see also Chapter 2).

GENOMIC STRUCTURE AND CHROMOSOMAL LOCALIZATION OF THE HUMAN AND MURINE HOMING RECEPTOR GENES

The overall mosaic structure of the human[41] and murine homing[42] receptors was consistent with the assembly of these glycoproteins from gene segments encoding separate functional domains. This hypothesis was borne out by the analysis of the structures of the human and murine homing receptor genes. These analyses demonstrated that the structure/function motifs found in these glycoproteins were indeed encoded by separate exons in the genome. Thus, it was found that the signal sequence, the lectin domain, the egf-like domain, each complement binding-like motif, and the transmembrane domain were all encoded by discrete exonic sequences. In addition, the initiator methionine codon was encoded by a separate exon, and the cytoplasmic domain was encoded by two short exons in both species. Interestingly, the intronic interruption site or "phase" of each exon (i.e., the nucleotide site in a given triplet codon that is interrupted by the intron) was the same as that found for analogous coding exons in other genes. Thus, other genes containing lectin-like, egf-like, and complement binding-like exons are all interrupted by introns at identical sites in the triplet codons, irregardless of what type of coding exon comes before or after the motif.[42,43] This finding is interesting in an evolutionary sense because it is consistent with the hypothesis that the homing receptor-encoding exons arose from primeval progenitor-type exons that were dispersed throughout the genome by exon shuffling followed by mutation and selection for certain functional attributes.

The high degree of sequence relatedness between the members of the LECCAM family was found to correlate well with their positions in the murine and human genome. By a series of chromosomal mapping studies, it was found that both the human and murine homing receptors mapped to syntenic regions of human and mouse chromosome 1.[41–44] This data was made even more interesting by the finding that the ELAM and GMP-140/

PADGEM genes also mapped to this same region in both human and mouse genomes. Pulse-field gel analysis demonstrated that these three genes were within approximately 200,000 bases of each other.[44] These data were consistent with the derivation of these genes by amplification of a single progenitor LECCAM/selectin gene whose progeny ultimately produced the three members of this family by mutation and selection. It is also interesting to note that although these genes physically map very close to one another, their regulation is highly specialized, so that each gene product is generated in dramatically different ways. This suggests that divergent gene regulatory pathways co-evolved with the individual LECCAM/selectin genes to allow for a greater level of regulation with respect to tissue type and temporal expression.

THE NATURE OF THE LIGANDS FOR THE HOMING RECEPTOR

From the data cited thus far, it seemed clear that the interaction between the homing receptor and its ligand was, at least in part, due to the recognition of a carbohydrate(s) by the lectin domain. Thus, the blocking of cell adhesion by carbohydrates, the calcium dependence of endothelial binding, and the abolition of such binding by sialidase combined with the calcium-dependent lectin homology discovered in the homing receptor glycoprotein sequence were all strongly consistent with this possibility.[11] In addition, it was shown that the isolated natural receptor could interact with carbohydrates such as PPME, further strengthening the notion that this receptor was, in fact, a carbohydrate-binding protein or lectin.[45,46] A further piece of evidence came when the epitope for the MEL-14 adhesion-blocking antibody was mapped. These data were consistent with previous data regarding the blocking of PPME binding by MEL-14 in that they showed that this antibody mapped to the N-terminus of the lectin (potential carbohydrate-binding) domain.[47] The mapping data, together with the adhesion-blocking ability of the antibody, were also consistent with a role for the lectin domain in mediating cell–endothelial adhesion by recognition of a specific carbohydrate(s) located on the endothelial cell surface.

In order to analyze the nature of the ligand(s) for the homing receptor, a reagent specific for this molecule had to be produced. Streeter and colleagues took a traditional approach to this problem by generating murine monoclonal antibodies directed against antigens specific for peripheral lymph node high endothelial venules.[48] One such antibody, termed *MECA 79,* specifically recognized an antigen(s) on this type of endothelium and also blocked the ability of lymphocytes to bind to these endothelial cells in frozen section assays. Analysis of the glycoproteins recognized by this monoclonal antibody demonstrated a relatively broad specificity, with a number of surface glycoproteins

reacting with the antibody. This result, coupled with the relatively low affinity and IgM isotype of the antibody, suggested that this monoclonal antibody may have been recognizing a carbohydrate epitope potentially involved in homing receptor–ligand interactions. A second, much more novel technology was utilized by Watson and colleagues to analyze the nature of the ligand(s) recognized by the homing receptor.[49] In this procedure, the extracellular domain of the murine receptor was ligated to the hinge, C_H2, and C_H3 motifs of the human IgG_1 molecule. This resulted in an artificial antibody-like molecule, termed the *homing receptor IgG chimera,* whose specificity for antigen was determined by the ability of the homing receptor to adhere to its ligand(s). In addition, the IgG region could be utilized for a number of purposes, including: 1) dimerization by virtue of the disulfide-bonded hinge region leading to enhanced avidity, 2) ease of purification due to the specific interaction of this region with protein A or G, and 3) the ability to analyze receptor–ligand interactions by immunohistochemistry or immunoprecipitation using readily available reagents. This reagent was found to recognize carbohydrates such as PPME, inhibit lymphocyte binding to endothelial cells in frozen section assays, and, perhaps most interestingly, to specifically stain peripheral lymph node high endothelial venules but not Peyer's patch venules. This staining was calcium-dependent, MEL-14 inhibitable, and blocked by carbohydrates such as fucoidin, consistent with a protein–carbohydrate interaction. These results demonstrated that this reagent could be utilized for histochemical studies to localize the ligand(s) for the homing receptor. In addition, it was consistent with the hypothesis that specific trafficking of lymphocytes to peripheral lymph nodes via the homing receptor-dependent pathway was due to the lack of ligand expression in the Peyer's patch. This exciting result suggested that region-specific ligand expression was indeed one of the mechanisms that directed specific leukocyte trafficking to various lymphoid sites.

While the above experiments suggested the usefulness of the homing receptor–IgG chimera as a reagent for analyzing the tissue distribution of the ligand(s) for the homing receptor, they only began to describe the biochemical nature of this ligand(s). In order to examine the potential carbohydrate nature of the ligand(s), tissue sections were treated with sialidase and were then stained with the homing receptor–IgG chimera. These results were consistent with previous data[21a,51] and demonstrated that removal of sialic acid resulted in an abolition of staining, again suggesting that the homing receptor–ligand interaction involved, in part, sialic acid recognition.[52–54] Imai and colleagues[55] carried the analysis of the ligand(s) much further by taking advantage of an interesting, although unexplained, observation made many years earlier. This early work demonstrated that high endothelial venules of peripheral lymph nodes rapidly incorporated large amounts of inorganic sulfate into a secreted macromolecule.[56] At the time, these investigators

proposed that this label was incorporated into a secreted glycolipid. Interestingly, autoradiographic analysis demonstrated that sulfate incorporation was found over the Golgi complex at early labelling times, also consistent with a potential glycoprotein localization of the label. Imai et al. reasoned that the tissue-specific localization of the sulfate labelling in addition to its localization over the endothelial venules suggested that this atom may have been incorporated into a ligand(s) for the homing receptor. Immunoprecipitation analysis of sulfate-labelled murine peripheral lymph nodes using the homing receptor IgG chimera revealed that a specific ~50-kD glycoprotein appeared to interact with this chimera. In addition, a ~90-kD protein was also immunoprecipitated by the receptor chimera, although it was labelled much less intensely. Surprisingly, analysis of the total sulfate-labelled material revealed that, in a short (~2 hours) pulse label, the ~50-kD protein band appeared to be the major sulfate-labelled molecule. In addition, and in agreement with the immunohistochemical results, the synthesis of these ligands was highly tissue-specific, with no ligand detected in other lymphoid tissues such as spleen or Peyer's patch. The interaction of both the 50-kD and 90-kD putative ligands with the homing receptor chimera was calcium-dependent and blocked by the MEL-14 antibody as well as by certain carbohydrates such as fucoidin and PPME. These results were consistent with the possibility that the interaction between the homing receptor and its ligand was, at least in part, due to protein (i.e., lectin domain)–carbohydrate interactions. This notion was further supported by experiments demonstrating that treatment of the isolated 50-kD protein with sialidase resulted in a ~5-kD decrease in molecular weight and a concomitant loss in ability to interact with the homing receptor chimera. This result was entirely consistent with all previous data concerning the role of sialic acid in adhesion mediated by the homing receptor. The carbohydrate attached to these ligands appeared to be all O-linked,[57] as treatment with N-glycanase showed no diminution of molecular weight. Referring to previous work by Streeter et al.,[48] analysis of the reactivity of the isolated ~50-kD ligand with MECA 79 revealed that this glycoprotein effectively interacted with this antibody, confirming previous data suggesting that this antibody recognized a component of the ligand for the homing receptor. Finally, protease digestion of the isolated ~50-kD band revealed that this component was a glycoprotein. In conclusion, this work revealed that the ligand(s) for the homing receptor were two sulfate-labelled, peripheral lymph node-specific glycoproteins whose interaction with this adhesive glycoprotein were in part mediated by carbohydrates, especially sialic acid. The relevance of the O-linkage of the sugar is presently not understood but may have to do with an enhanced presentation of the relevant carbohydrate residues to the homing receptor lectin domain.[57] A similar, although much less clear cut, analysis of the homing receptor ligand(s) has recently been presented.[58]

STRUCTURE AND FUNCTION OF HOMING RECEPTOR DOMAINS

While the data concerning calcium dependence, carbohydrate and antibody blocking, and sialidase sensitivity all argued strongly for a role for the putative carbohydrate-binding or lectin domain in adhesion mediated by the homing receptor, it is possible that other domains are involved with cell adhesion as well. An interesting phenomenon was noted when it was reported that recognition of the lectin-localized MEL-14 epitope appeared to be dependent upon the inclusion of the egf-like domain.[47] This phenomenon has also been noted for the ELAM molecule as well as for the human homologue of the homing receptor (Tedder,T., personnal communication). Assuming that the epitopes for these various antibodies are located exclusively within the lectin domain, these results are consistent with the interpretation that the egf domain has an effect on the overall conformation of the lectin domain. In addition, the work of Siegelman et al.[50] has suggested a potentially even more important role for the egf-like domain in cell adhesion. These authors demonstrated that the epitope recognized by the allotypic antibody LY22 was located in the egf domain of the homing receptor, where a single amino acid polymorphism was found to be responsible for the generation of this allotypic response. These authors went on to show that this antibody could effectively block the binding of lymphocytes to peripheral lymph node endothelium, suggesting that the egf-like domain may be directly involved with cell adhesion. In addition, they showed that the binding of this antibody enhanced the binding of the carbohydrate PPME to the homing receptor. Assuming that this antibody actually binds exclusively to the egf-like domain, this enhancement is consistent with a modification of the conformation of the lectin domain by antibody-induced perturbation of the egf domain. Thus, these results may be interpreted to agree with previous data indicating a role for the egf domain in lectin domain structure, as well as suggesting a more direct role for the egf-like domain in cell adhesion. It must be cautioned, however, that the enhancement of binding to PPME, an artificial binding substrate, cannot be taken to mean a necessary enhancement of binding to the natural ligand, so that this apparent modification of the lectin domain by antibody binding may have had an adverse effect on recognition of the endothelial ligand. It is therefore conceivable that a conformational modification of the lectin domain may have been responsible for the inhibition of adhesion by the LY22 antibody. The direct role of the egf-like domain in cell adhesion, therefore, awaits further experimentation.

While these data suggested several potential roles for the lectin and egf domains of the homing receptor, the function(s) of the complement binding-like motifs were still not understood. The somewhat lower degree of homology between the human and murine forms of these domains compared to the lectin and egf-like motifs suggested a less stringent role for these regions in

homing receptor function. The identity of the two complement-binding motifs in the murine homing receptor at the nucleotide level suggested a critical need for two of these repeats, although the fact that the human motifs are not identical to one another suggests that they need to have homology but not identity for appropriate function. In order to analyze the role(s), if any, of these motifs in lectin domain function, homing receptor-IgG chimeras were produced with deletions of these duplicated domains.[59] The first surprising results obtained with this deletion mutant was that reactivity with the MEL-14 antibody dropped precipitously, suggesting a role for these motifs in lectin domain structure potentially analogous to that suggested for the egf domain. Not surprisingly, this loss of MEL-14 reactivity was accompanied by a loss of carbohydrate (PPME)-binding capacity, suggesting that the diminution of MEL-14 binding activity was probably due to a perturbation of the lectin domain conformation. This mutant was also unable to recognize the endothelial ligand for the homing receptor in cell blocking, immunohistochemical staining, and immunoprecipitation experiments. These results were all consistent with a role for the complement-binding repeats in appropriate lectin domain function. While a direct conformational interaction between the lectin and complement-binding motifs may be one potential explanation for this finding, a more likely one is that these domains serve to form oligomeric complexes that increase avidity as well as serve to induce appropriate lectin conformation. Other type C lectins appear to show a preference for oligomer formation, and at least one other selectin, ELAM, appears to form oligomeric complexes when expressed as a secreted molecule.[60] The individual domains of the homing receptor appear to act in complex coordinated manners that are still not fully understood.

THE NEUTROPHIL HOMING RECEPTOR: A MEDIATOR OF NEUTROPHIL ROLLING *IN VIVO*

While it seemed clear that the function of the homing receptor of lymphocytic cells was to enable them to efficiently traffic to peripheral lymphoid organs, the location of this receptor on other leukocytes, such as neutrophils,[61] was somewhat less clear. Cells such as neutrophils do not ordinarily traffic to peripheral lymph nodes but instead are found in acute inflammatory sites Thus, it was thought that the neutrophil homing receptor might function as a mediator of neutrophil adhesion during some step of acute inflammation. This possibility was supported by early experiments that demonstrated that acute neutrophil-mediated inflammation in a number of *in vivo* inflammatory models could be blocked by the MEL-14 antibody. Later experiments revealed that removal of the homing receptor from the lymphocyte surface by either activation (see below) or by mild trypsin digestion resulted in cells

that were unable to appropriately migrate to inflammatory sites,[62,63] again consistent with a role for this adhesion molecule in inflammation. While these results implied a function for the homing receptor in neutrophil inflammatory processes, the experiments were criticized because either the mere binding of the antibody to the neutrophil surface might have had deleterious effects on neutrophil function that were not related to adhesion or the activation and/or proteolysis of the neutrophil may have had more pleiotropic effects than mere removal of the homing receptor. Another approach was taken by Watson et al.[64] when they examined the ability of the previously described homing receptor–IgG chimera to inhibit acute neutrophil-mediated inflammation in the peritoneal inflammatory model. In this model, inoculation of thioglycollate into the mouse peritoneum results in a rapid and profound neutrophil influx. These investigators found that intravenous administration of ~30 microgram per ml of the homing receptor chimera to mice before the induction of the peritoneal inflammatory response resulted in a profound inhibition of the ability of neutrophils to traffic to the peritoneum. The chimera also resulted in a significant, but lesser, degree of inhibition of the trafficking of lymphocytes to peripheral and mesenteric, but not to Peyer's patch, lymph nodes. As little as ~3 micrograms per ml gave a significant inhibition of neutrophil influx. In addition, these investigators found that the inhibition was almost complete at two hours after the induction, but appeared to be less significant at four hours. The interpretation of this work was that the soluble adhesion chimera competitively inhibited the adhesive interaction between the neutrophil homing receptor and its ligand(s) on the endothelial cell surface adjacent to the inflammatory site. These results were consistent with a significant role for the neutrophil homing receptor in acute inflammatory responses, and also implied that a carbohydrate-like ligand similar to that found in the peripheral lymph node may have been responsible for neutrophil trafficking to acute inflammatory sites.

The above data, while indicating a critical role for the homing receptor in neutrophil-mediated inflammation, did not directly identify what this specific task(s) was. For example, the homing receptor may have been involved in the efficient high avidity contact between neutrophils and the endothelium in a manner similar to that proposed for the interaction(s) between the lymphocyte and the postcapillary venule of the peripheral lymph node.[65] Alternatively, it was possible that this adhesion molecule was involved in a physiological function first identified about 100 years ago and termed *neutrophil rolling*. Using the so-called intravital microscopy technique, examination of postcapillary venules during the early stages of acute inflammation revealed that neutrophils interacted with the endothelium in a low affinity manner, such that the cells were observed to roll along the endothelium at a rate much slower than the rate seen for the unattached cells in the

circulation.[66,67] This rolling episode was found to be the precursor for the later higher affinity events that resulted in the complete arrest of neutrophil motion followed by a change in the shape of the cell from a rounded to flattened morphology and finally by diapedesis across the endothelial barrier into the inflammatory site. Thus, it could be concluded that neutrophil, and perhaps other leukocytic cell, inflammation appeared to be a multistep phenomenon consisting of initial low-affinity interactions followed by higher-affinity adhesive events.

In order to examine the possibility that the neutrophil homing receptor was involved in leukocyte rolling, the same technique that was used to originally describe this phenomenon was applied, using more modern techniques and reagents. In these studies, Ley et al.[68] utilized intravital videomicroscopy of rat mesenteric postcapillary venules to examine the effects of the homing receptor chimera and a polyclonal antibody directed against the homing receptor on neutrophil rolling *in vivo.* An adjacent upstream venule was cannulated and injected with various solutions, then videomicroscopy of the downstream venule was utilized to quantitate the numbers of neutrophils rolling during a given time period. These investigators found that injection of a 100 microgram per ml solution of the murine homing receptor chimera inhibited neutrophil rolling by ~85%, while injection of a human CD4-IgG chimera gave no such inhibition. In addition, injection of a polyclonal rabbit antibody directed against the murine homing receptor also inhibited neutrophil rolling by ~80%, while a pre-immune serum showed no such effect. The authors also examined the temporal effects of inhibitor administration by investigating the rate that normal leukocyte rolling recurred after the termination of a constant perfusion of either the homing receptor chimera or polyclonal anti-homing receptor antibody. These studies showed recurrence of normal neutrophil rolling approximately 15 to 20 seconds after termination of inhibitor perfusion. In the case of the chimera, these results were consistent with a low-affinity interaction between the homing receptor chimera inhibitor and the endothelial ligands(s) for this adhesion molecule, so that the inhibitor dissociated from the ligand relatively quickly. This type of low-affinity interaction would be exactly as expected for the rolling phenomenon, where adhesive interactions would be expected to be made and broken with quite regular frequency. These elegant experiments were thus consistent with a critical function for the neutrophil homing receptor in leukocyte rolling near sites of chronic inflammation. In addition, they served to explain the *in vivo* blocking results of Watson et al.[64] and suggested that inhibition of neutrophil rolling is accompanied by a concomitant inhibition of tissue inflammation. In conclusion, these studies demonstrated that neutrophil rolling is, at least in part, mediated by the homing receptor and that the rolling phenomenon is a critical precursor to other adhesive and migratory aspects of the inflammatory response.

REGULATORY ASPECTS OF HOMING RECEPTOR FUNCTION

The regulation of homing receptor function is apparently accomplished using a number of different mechanisms. Perhaps the most basic is the regulation of cell type and developmental time of the expression of this adhesive glycoprotein.[31,33,36] In the case of the lymphocytic population, expression of the homing receptor appears to await entry of these cells from the bone marrow into the thymic compartment. The antigen is then expressed during thymic development so that lymphocytes released from this compartment appropriately traffic to peripheral and mesenteric lymph nodes. It may be assumed that expression of the glycoprotein before thymic education would result in trafficking of unselected bone marrow lymphocytes directly to peripheral lymphoid compartments, with the possibility that auto-immune like syndromes would develop. Thus, the highly regulated expression of this adhesion molecule in these cells appears to insure appropriate thymic education before release of the cells to the periphery. In contrast to this situation, expression on myeloid cells appears to occur much earlier in bone marrow development. In humans, it has been shown that expression occurs on very early myeloid precursor cells, suggesting other potential roles for this molecule in adhesion and trafficking. The early expression on myeloid cells is expected in view of the fact that these leukocytic cells are released directly into the circulation so that they can function immediately. In addition, the high turnover rate of the granulocyte population is consistent with a rapid release of neutrophils whose functional capacity to invade inflammatory sites is pre-existent. Thus, it may be concluded that regulation of expression of the homing receptor on various cell types during hematopoiesis is a key step in appropriate function of these cells during peripheral trafficking.

A second interesting aspect of homing receptor regulation concerns the ability of cells to rapidly shed the glycoprotein from their surfaces.[35,36,69–76] In the case of neutrophils, this shedding appears to occur within seconds of a number of physiologic stimuli, many of which appear to be involved with neutrophil activation. The release from lymphocytes, especially in response to antigenic stimuli, appears to occur with much slower kinetics. Interestingly, the regulation of release of the homing receptor from the neutrophil surface is a mirror image of the activation of the adhesive integrins of the beta 2 type (i.e., CD11/CD18). Thus, loss of the homing receptor after neutrophil activation is accompanied by a concomitant upregulation of expression and presumed adhesive capacity mediated by the beta 2 integrins. This regulation, when viewed in light of previous data concerning the function of the homing receptor in neutrophil rolling, suggests a model where rolling neutrophils, once activated by factors near the inflammatory site, lose their surface homing receptors and bind with high affinity to the endothelium by virtue of the CD11/CD18 integrin complex. The loss of the surface homing

receptor might insure that inappropriate release of the neutrophil from the endothelium would not result in activated, and potentially damaging, neutrophils from rolling and attaching to other noninflammatory sites.

The mechanism by which this rapid loss occurs is quite interesting. The slightly smaller size of the released material suggests that the shedding appears to be mediated by proteolysis at a site very close to the transmembrane anchor site of the receptor. The work of Camerini et al.[28] initially suggested that there may be two forms of the receptor, one with the accepted transmembrane anchor and a second with a glycophospholipid (GPI) anchor. This second form was hypothesized based upon a second cDNA clone with a potential GPI anchor signal (hydrophobic C-terminal domain) and a very limited transient expression experiment that seemed to demonstrate a lipid-anchored form of the molecule. However, a variety of data, including genomic structural analysis[41,42] that is consistent with a lack of alternative RNA splicing, lack of phospholipase C cleavage of the receptor on a number of cells, normal expression of the receptor on patients with paroxysmal nocturnal hemoglobinuria (PNH—a somatic mutation that prevents GPI linkage), and the absence of any other mRNAs by polymerase chain reaction analysis,[41] argue that the second message seen by Camerini et al. may have been a cDNA cloning artifact. The nature of the protease that mediates this cleavage may be unique. The high degree of conservation of the transmembrane anchor regions between the human and murine receptors[27] suggests that the protease may be a membrane-anchored one that adheres to the homing receptor specifically through this conserved domain. The tight linkage of the protease to the receptor is supported by data demonstrating that inclusion of a variety of protease inhibitors in the media of cells expressing the receptor does little to inhibit the shedding of the molecule. Its potential linkage with cell signalling elements is supported by the finding that agonists of protein kinase C activity appear to induce rapid cleavage of the receptor from the cell surface, while inhibitors of this kinase appear to decrease the rate of shedding.[71,74] Thus, the proteolytic cleavage of this adhesion receptor and resultant shedding appear to be highly regulated functions.

A third important aspect of homing receptor regulation revolves around an apparent change in the avidity of the receptor for carbohydrate and endothelial ligand binding. The work of Spertini et al.[77] revealed that appropriate activation of lymphocytes or other cells resulted in an increased affinity of the homing receptor for the carbohydrate, PPME. This enhanced affinity was accompanied by an increased binding of the activated cells to the endothelium of peripheral lymphoid tissue in the frozen section assay. These results may be interpreted in the context of other adhesion molecules, such as the beta 2 integrins, which also appear to show an enhanced avidity for their ligand(s) after cell activation. In the case of the integrins, and presumably the homing receptor as well, the enhanced affinity is due to a conformational

change in the protein. This conformational change may be in the overall three-dimensional structure of the protein or it may be due to oligomerization of the receptors with a resultant enhanced binding avidity. This level of regulation thus allows for a cell type-specific activation of the adhesion mediated by this receptor, as the enhanced avidity would be dependent upon the type of activation and the spectrum of receptors (i.e., antigen-specific, chemotactic) found on the cell surface. This type of activation would thus partially explain why only specific types of cells appear to traffic to various sites, in spite of the fact that a number of nontrafficking cells also possess cell surface homing receptor. Thus, the specific trafficking of lymphocytes to peripheral lymphoid tissue may be accomplished by lymphocyte-specific activation signals in these organs, while the rapid and specific early trafficking of neutrophils to sites of acute inflammation may be accomplished by a divergent set of neutrophil-specific activators produced at these acute inflammatory sites. The possibility that this activation event is dependent upon protein kinase C-mediated phosphorylation of the cytoplasmic domain has been previously alluded to and awaits experimental confirmation.

A final, although unproven, level of regulation may involve signalling mediated by the homing receptor to activate cell motility and/or shape. A potentially significant set of data in this regard suggests that exposure of lymphocytes to pertussis toxin, an inhibitor of G protein-mediated cell signalling, appears to profoundly affect lymphocyte trafficking to peripheral lymphoid organs.[78] Indeed, animals given pertussis toxin show a high level of lymphocytosis, suggesting that these cells are incapable of migrating through the peripheral lymphoid organ pathway, thus resulting in an increase in the levels of circulating lymphocytes. While these results may be interpreted in a number of different ways, one possible interpretation is that the homing receptor interacts with one or more G proteins on the cytoplasmic side, perhaps through protein–protein interactions in the highly conserved transmembrane domain region, so that contact of the homing receptor with the endothelium activates a cellular signalling cascade that results in changes in cytoskeletal elements and resultant cell motility into the lymphoid organ. While the veracity of this proposal remains to be determined, one report suggests that an antibody directed against the human homing receptor can inhibit B-cell differentiation, consistent with a potential signalling mechanism of B cell homing receptor.[79]

SUMMARY

The homing receptor is the paradigm for a family of inflammatory adhesion molecules that mediate leukocyte–endothelial binding through protein–carbohydrate interactions. The homing receptor appears to have a critical function in normal peripheral lymph node trafficking as well as in acute

inflammatory responses mediated by neutrophils. It remains to be seen whether this adhesion molecule is involved in other inflammatory conditions, particularly chronic inflammatory syndromes such as arthritis or autoimmune disease. The involvement of this adhesion receptor in acute neutrophil-mediated syndromes suggests potential clinical uses for inhibitors directed against the adhesive functions of the homing receptor. Such inhibitors, including soluble receptor–immunoglobulin chimeras such as those described previously or carbohydrate-like molecules based upon the naturally occuring ligands,[80] will undoubtedly be clinically tested in the very near future. It is hoped that the knowledge produced from basic investigations into the molecular and cellular biology of the homing receptor may ultimately result in efficient antiinflammatory compounds for a number of currently untreatable clinical conditions.

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